#19 3/12/02

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

SAVERIO CARL FALCO ET AL. CASE NO.: BB1205 US NA

APPLICATION NO.: 09/464,528 GROUP ART UNIT: 1646

FILED: DECEMBER 15, 1999 EXAMINER: C. COLLINS

FOR: S-ADEOSYL-L-METHIONINE SYNTHETASE PROMOTER AND ITS USE IN EXPRESSION OF TRANSGENIC GENES IN PLANTS

Assistant Commissioner For Patents
Washington, DC 20231

Sir:

Declaration Pursuant to 37 CFR §1.132

I, Zhongsen Li, am a citizen of People's Republic of China and a permanent resident of the United States of America, residing at 24 Forest Creek Drive, Hockessin, Delaware 19707, United States of America, and I declare as follows:

1. I am one of the above-identified co-inventors named in this application. I am a graduate of Lanzhou University, People's Republic of China with a B.A. degree granted in 1983 in biology. I received a Ph.D. degree in 1992 from the Institute of Botany, Chinese Academy of Sciences in plant cell biology and my second Ph.D. degree in 1997 from Texas A&M University in molecular biology. From 1997 to 1998 I was a postdoctoral fellow at E. I. du Pont de Nemours and Company. I have been employed by E. I. du Pont de Nemours and Company since 1999 directing and conducting research in plant genetic engineering.

2. I have reviewed the Office Action dated July 5, 2001 and am aware that this declaration is being submitted to illustrate work which I, or those working under my guidance and direction have done, in further demonstrating that an additional DNA fragment (SEQ ID NO:14) will function as a promoter in a transgenic cell or plant.

3. Two different soybean SAMS DNA fragments, containing the nucleotides sequences of SEQ ID NO:6 and 14, were shown to have promoter activity in transgenic soybean cells. Although data was previously provided in the specification for SEQ ID NO:6 functioning as a promoter in *Arabidopsis* and corn, additional data is provided herein with respect to soybean. The plasmid DNA constructs used are described in TABLE 1.

TABLE 1

Plasmid DNA	Promoter	Coding Region	Terminator
pZSL11	1.3-kb SAMS (SEQ ID NO:6)	GUS	NOS
pZSL12	2.1-kb SAMS (SEQ ID NO:14)	GUS	NOS
pZSL13	1.3-kb SAMS (SEQ ID NO:6)	Soybean ALS*	Soybean ALS
pZSL14	2.1-kb SAMS (SEQ ID NO:14)	*Arabidopsis* ALS*	*Arabidopsis* ALS

* Mutant soybean and Arabidopsis Acetolactate Synthase (ALS) genes were used, that encode ALS enzymes resistant to sulfonylurea herbicides.

4. Plasmid pZSL11 contains the 1.3-kb SAMS promoter (SEQ ID NO:6) operably linked to the GUS reporter gene (Jefferson (1987) *Plant Mol. Biol. Rep.* 5:387-405), and the NOS terminator (Depicker *et al.* (1982) *J. Mol. Appl. Genet.* *1*:561-570). The construction of pZSL11 is described in Example 5 of the specification. The nucleotide sequence of the {1.3-kb SAMS promoter – GUS – NOS} region corresponds to SEQ ID NO:18.

5. Plasmid pZSL12 was made by replacing the 5' region of the 1.3-kb SAMS promoter in pZSL11 with a longer SAMS genomic DNA from pZSL10, a plasmid DNA containing an 2335-bp SAMS genomic DNA cloned in pBluescript KS. The 1675-bp XhoI(blunt-ended with *E. coli* DNA polymerase I Klenow fragment)/BamHI fragment from pZSL10 was transferred into pZSL11, to replace the corresponding 809-bp XbaI(blunt end with *E. coli* DNA polymerase I Klenow fragment)/BamHI fragment. The resulting plasmid, pZSL12, has a 2.1-kb SAMS promoter (SEQ ID NO:14) which is 869-bp longer than the 1.3-kb SAMS promoter in pZSL11. The nucleotide sequence of the {2.1-kb SAMS promoter – GUS – NOS} region from pZSL12 is shown in Figure 1.

6. Plasmid pZSL13 was made by replacing the GUS gene and NOS terminator in pZSL11 with a DNA fragment containing a soybean mutant ALS

coding region and its 3'-UTR (UnTranslated Region). The mutant soybean ALS gene encodes an enzyme that is resistant to sulfonylurea herbicides. The nucleotide sequence of the {1.3-kb SAMS promoter – mutant soy ALS – soy ALS 3'-UTR} region in pZSL13 is shown in Figure 2. Plasmid pZSL14 was made by linking the 2.1-kb SAMS promoter from pZSL12 to a DNA fragment containing a mutant *Arabidopsis* ALS gene and its 3'-UTR. The mutant *Arabidopsis* ALS gene encodes an enzyme that is resistant to sulfonylurea herbicides. The nucleotide sequence of the {2.1-kb SAMS promoter – mutant *Arabidopsis* ALS – *Arabidopsis* ALS 3'-UTR} region in pZSL14 is shown in Figure 3. Mutant plant ALS genes encoding enzymes resistant to sulfonylurea are described in U.S. Patent 5,013,659 (1991), "Nucleic acid fragment encoding herbicide resistant plant acetolactate synthase".

7. Soybean transformation was performed as follows:

Soybean embryogenic suspension cultures were transformed with the GUS-containing plasmids, pZSL11 and pZSL12, by the method of particle gun bombardment using procedures know in the art (Klein et al. (1987) *Nature* (London) *327*:70-73; U.S. Patent No. 4,945,050; Hazel, et al. (1998) *Plant Cell Rep 17*:765-772; Samoylov, et al. (1998) *In Vitro Cell Dev Biol - Plant 34*:8-13). The selective agent used was hygromycin (50 mg/mL). In addition, 0.6 μm gold particles were used instead of 1.0 μm particles. Soybean embryogenic suspension cultures were transformed with plasmids pZSL13 and pZSL14, each containing a mutant ALS gene, by a similar procedure with the following modifications.

Stock tissue for these experiments were obtained by initiation of soybean immature seeds. Secondary embryos were excised from explants after 6 -8 weeks on media. Secondary embryos were placed on media for 7-9 days under ~80$\mu Em^{-2}s^{-1}$ light intensity. Tissue was dried on Whatman #2 filter paper then moved to a prebombardment osmotic treatment (media containing 0.25M mannitol and 0.25M sorbitol) for 4 hours under ~80$\mu Em^{-2}s^{-1}$ light intensity. After 4 hours, tissue was moved to an empty 60X15 mm petri dish for bombardment. Approximately 10mg of tissue (10-15 clumps of 1-2mm size) were used per plate bombarded.

After bombardment, tissue was moved to media for an overnight incubation at ~80$\mu Em^{-2}s^{-1}$ light intensity. Tissue was divided in half and placed in liquid media for selection. For selection of transformed cells containing the mutant ALS gene (pZSL13 and pZSL14), the selective agent used was a sulfonylurea (SU) compound with the chemical name Benzenesulfonamide, 2-Chloro-N-///-4-methoxy-6 methyl-1,3,5-Triazine-2-YL/ Aminocarbonyl (common names: W4189 and chlorsulfuron). The concentration of SU used was 90 ng/ml. SU was applied

one week after bombardment and continued for six weeks, with a fresh media and SU change once a week. After six weeks, events were isolated and kept at 90 ng/ml concentration for another 4-6 weeks. Total time in SU was 8-12 weeks.

After selection, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue was removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Suspension cultures were subcultured and maintained as clusters of immature embryos and also regenerated into whole plants by maturation and germination of individual somatic embryos.

8. SAMS promoter activity in transgenic soybeans was determined as follows:

Soybean embryogenic suspension cells, transformed with either pZSL11 or pZSL12, were assayed for GUS activity by the histochemical staining procedure described in Example 5, page 22, of the specification. From the results of this assay, it was observed that both the 1.3-kb (SEQ ID NO:6) and the 2.1-kb (SEQ ID NO:14) fragments from the SAMS gene displayed promoter activity (Figure 4).

Soybean plants were regenerated from embryogenic suspension cells transformed with either pZSL11 or pZSL12. The results of GUS histochemical staining of pZSL11 transformed soybean tissues (embryogenic suspension cells, leaf, stem and root) are shown in Figure 5. These results indicate promoter activity for the 1.3-kb (SEQ ID NO:4) fragment of pZSL11 in each of these cell types (Figure 5). Similar results were obtained for the 2.1-kb (SEQ ID NO:14) fragment of pZSL12.

The 1.3-kb and 2.1-kb SAMS fragments in pZSL13 and pZSL14, respectively, were also used to drive expression of the SU-resistant mutant ALS genes from soybean (pZSL13) and *Arabidopsis* (pZSL14). Transformed soybean cell lines were selected using the SU herbicide, as described above. Transgenic soybean cell lines containing either plasmid DNA were obtained, demonstrating that both SAMS fragments functioned as promoters in embryogenic suspension cells.

Soybean plants, transformed with either pZSL13 or pZSL14, were tested for tolerance to SU herbicide. A spray solution was made containing 60 grams of Thifensulfuron-methyl active ingredient per hectare and 0.25% wt/wt of AL-2999 nonionic surfactant. Thifensulfuron-methyl is the active ingredient in the two DuPont sulfonylurea herbicides, Harmony GT® and Pinnacle®. Either Harmony GT® or Pinnacle® can be used as the source of this sulfonylurea for the spray test. AL-2999 is a nonionic surfactant, obtainable as Atplus UCL 1007® from Uniqema. This mixture was evenly sprayed onto the soybean plants at the 1st or

2nd trifoliate stage of development. After waiting approximately two weeks the results were scored. All wild-type plants (or plants lacking the SAMS:mutant ALS transgene) were dead (negative control), all plants from commercially available STS® (Sulfonylurea Tolerant Soybean) seeds were alive (positive control), and plants containing the SAMS:mutant ALS transgene from either pZSL13 or pZSL14 also survived. Consequently, either the 1.3-kb (SEQ ID NO:6) or the 2.1-kb (SEQ ID NO:14) fragment from the SAMS gene can drive expression of the mutant ALS gene at levels sufficient to provide tolerance to SU.

9. In summary, both the 1.3-kb (SEQ ID NO:6) and the 2.1-kb (SEQ ID NO:14) fragments from the SAMS gene functioned as promoters in transgenic soybean. Promoter activity was observed in multiple cell types (embryonic suspension cells, leaf, stem and root). In addition, promoter activity was sufficient to drive functional expression of both a screenable marker (GUS) and a selectable marker (mutant ALS) gene.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Zhongsen Li

Zhongsen Li

11/30/2001

Date